Supporting Information: Methods

Patients

Both patients (patient 1: male, 72 years, post-operative survival 39 months; patient 2: female, 65 years, post-operative survival 5 months) had mixed intestinal/diffuse gastric cancer with chronic gastritis in adjacent tissue; patient 1 had 1-6 regional lymph nodes affected and no distant metastasis; patient 2 had >15 regional lymph nodes affected with distant metastases. Myofibroblasts were obtained from the primary tumor and immediately adjacent tissue,[1] cultured as previously described and used between passages 3 and 10.[2, 3] All myofibroblasts had previously been shown to be positive for α-smooth muscle actin and vimentin, and negative for desmin and pancytokeratin.[3]

Methionyl COFRADIC

Guanidinium hydrochloride was added to each sample to a final concentration of 0.8 M followed by 200 µL 1 M Tris-HCl pH 8.7. Iodoacetamide was added to a final concentration of 16 mM, and tris(2-carboxyethyl)phosphine to a final concentration of 8 mM. Samples were vortexed and incubated at 37°C for 30 min. Samples were then desalted using NAP-5 columns (GE Healthcare) and eluted in 1 mL of 50 mM triethylammonium bicarbonate buffer pH 8.5 (Fluka). Equal amounts of light and heavy labeled samples were then pooled and 5 µg of sequencing-grade modified trypsin (Promega, Madison, WI, USA) added and samples were incubated at 37°C for 18 h. The digested samples were acidified by addition of 50 µL 10% w/v acetic acid and dried to about 100 µL by vacuum centrifugation. The pH of the samples was measured and, when necessary, adjusted using TFA in order to ensure that column retention times will not be influenced by pH. The samples were run on an Agilent 1100 HPLC system with a Zorbax 300SB-C_{18} column (Agilent) at a flow rate of 80 µL/min, and fractions collected every minute between 20 and 80 min of the run. Solvent A was 10 mM ammonium acetate, 2% v/v acetonitrile, pH 5.5, and solvent B was 10 mM ammonium acetate, 70% v/v acetonitrile, pH 5.5 (Supporting Information Methods Table SM1). Following injection of the samples onto the column, a 10 min isocratic run with solvent A was applied, followed by a linear binary gradient from solvent A to solvent B over 100 min, a 10 minute isocratic wash with solvent B and finally a 5 min linear gradient back to solvent A. Collected fractions were pooled as shown in Supporting Information Methods Table SM1 to give 15 samples, which were then dried by vacuum centrifugation. Each sample was resuspended in 90 µL of 0.5% w/v acetic acid, 5 µL of which was analysed by

LC-MS/MS. The remainder of the samples were then re-run on the reverse-phase HPLC under the same conditions as the primary run, with the exception of a pre-incubation step with 0.1% v/v hydrogen peroxide at 30°C for 30 min in order to oxidise methionine residues. Blank runs were performed after every 5 samples. Secondary fractions were collected according to the strategy shown in Supporting Information Methods Table SM2, with 24 secondary fractions collected during each of the 15 runs. These secondary fractions were pooled such that, within each of the 15 sets of 24 secondary fractions produced, every sixth fraction was pooled to give 6 pooled samples per set of 24 secondary fractions, and dried by vacuum centrifugation. This produced a total of 90 fractions for analysis. The samples were then resuspended in 20 µL of 2% v/v acetonitrile and analysed by LC-MS/MS (Supporting Information Methods Fig. SM1).

N-terminal COFRADIC

Equal protein amounts of light and heavy labelled samples were combined and guanidinium hydrochloride was added to a final concentration of 4 M. Iodoacetamide was added to a final concentration of 10 mM, and tris(2-carboxyethyl)phosphine to a final concentration of 5 mM. Samples were vortexed, incubated at 37°C for 30 min and desalted using a NAP-5 column (GE Healthcare) in 850 µL 1.4 M guanidinium HCl in 50 mM sodium phosphate (pH 8) buffer. An N-hydroxysuccinimide ester of trideutero-acetate (D3-NHS) was added to the samples in a ratio of 150 g D3-NHS to 1 g protein and samples were incubated for 120 min at 30°C. Hydroxylamine was added in 4-fold molar excess over D3-NHS followed by incubation for 15 min at 30°C in order to revert partial O-acetylation of hydroxyl groups. Glycine was then added in 2-fold molar excess over D3-NHS in order to quench excess any remaining D3-NHS reagent. The samples were desalted again, this time using a NAP-10 column (GE Healthcare) and eluted in 1.3 mL 10 mM NH_4HCO_3, incubated at 95°C for 5 min, then transferred to ice for 5 min before adding sequencing-grade modified trypsin (Promega, Madison, WI, USA) in a ratio of 1/100, w/w (trypsin/protein) and incubated for 18 h at 37°C. Samples were then dried by vacuum centrifugation, redissolved in 300 µl SCX solvent A (10 mM sodium phosphate, 50% v/v acetonitrile, pH3.0) and further acidified to pH 3 with careful addition of 50% v/v acetonitrile containing 0.1%, 0.5% or 1% v/v TFA as required, such that the final TFA concentration is less than 0.08% v/v in a final volume of 1 ml. SCX enrichment was performed on an AccuBond cartridge (Agilent). The cartridge was rinsed with 5 ml of double distilled water and then pre-equilibrated with 10 mL of SCX solvent A. Samples (1 mL) were loaded onto the

column and the run-through fraction was collected followed by a washing step with 5 mL SCX solvent A and 2 mL 5 mM NaCl in SCX solvent A. Samples were thus recovered in a total volume of 8 mL and the pH was set back to 7 by addition of 5 M NaOH after which peptides were dried completely by vacuum centrifugation. A pyro-glutamate removal step was then performed utilising the pGAPAse from the TagZyme® kit (Qiagen) which was purified by Ni^{2+}-immobilised metal ion chromatography (IMAC). This resulted in the pGAPase being dissolved in 25 µl 20 mM ammonium bicarbonate, 500 mM immidazole, and 25 µL of this solution was further diluted in 1.25 µl 800 mM NaCl, 1 µl 50 mM EDTA and 11 µl 50 mM fresh cysteamine. The pGAPase was activated by incubation for 10 min at 37°C (in a total volume of 38.25 µl). The dried peptide samples were redissolved in 27 µl of 100 mM sodium phosphate and the activated pGAPase was added as well as 25 µl Q-cyclase (Qiagen). The whole mixture (in a total volume of 125 µl) was incubated for 60 min at 37°C, acidified with 0.5% acetic acid to pH 5, and then centrifuged at 16,000g to remove insoluble components. The samples were then run on an Agilent 1100 HPLC system with a Zorbax 300SB-C_{18} column (Agilent) under the same conditions as used in the Methionyl-COFRADIC method, with the methionine oxidation step performed immediately before the primary run (Supporting Information Methods Table SM2). According to the N-terminal COFRADIC protocol 15 primary 4-minutes fractions were collected at equal intervals between 20 and 80 minutes. The primary fractions were dried by vacuum centrifugation and resuspended in 20 µL 2% v/v pyridine in H_2O. Every fraction was again dried and resuspended in 50 µL of 50 mM sodium borate pH 9.5, and 15 nmol of 2,4,6-trinitrobenzenesulphonic acid (TNBS) was added to each fraction followed by incubation at 37°C for 30 min. The addition of TNBS and incubation was repeated a further three times and generated highly hydrophobic conjugates exclusively on free α-amine groups, which will be present at the N-terminus of all peptides except for those which have been blocked by in vivo acetylation, i.e. the N-terminal peptides. Upon TNBS modification every primary fraction was acidified by addition of 3 µL acetic acid, and centrifuged for 10 min at 16,000 x G to remove insoluble material. A second reverse-phase HPLC run was then performed under the same conditions as the primary run, with each of the 15 primary fractions run separately and each producing 12 secondary fractions. Those fractions which could be seen to contain peptides which had not shifted following the TNBS reaction were collected for LC-MS/MS analysis, since these were enriched for N-terminal peptides (Supporting Information Methods Fig. SM2). Corresponding secondary fractions from primary

fractions with a 12-minute time interval were pooled to generate 36 samples which were dried and resuspended in 20 µL 2% v/v acetonitrile for LC-MS/MS analysis.

SILAC labeling

For Patient 1 COFRADIC experiments, CAMs were heavy labeled, and ATMs were light labeled. For patient 1 non-COFRADIC experiments, this labeling was reversed. This allowed for the identification and removal of some serum contaminant proteins from the datasets, which appeared as light labeled only in both sets of experiments. For patient 2 experiments, CAMs were heavy labeled and ATMs were light labeled.

In vivo imaging

Gastric cancer cells (MKN45, 5 x 10^5) were injected either alone or with CAMs (1 x 10^5 MKN45 + 5 x 10^4 CAMs) on the left flank of the mice. Tumor size was monitored three times per week for 6 weeks. Once the tumor reached 1.2 cm mean diameter, or demonstrated early signs of ulceration, 100 µl MMPSense 750 FAST™ which produces a fluorescent signal upon cleavage by MMPs was administered by IV tail vein injection and fluorescence molecular tomography (FMT) imaging was performed in the 745 nm channel using the VisEn FMT 2500 LX system 24 h later. Fluorescence from the tumor was quantified using TrueQuant software.

5 References

1. Wu, K. C.; Jackson, L. M.; Galvin, A. M.; Gray, T.; Hawkey, C. J.; Mahida, Y. R., Phenotypic and functional characterisation of myofibroblasts, macrophages, and lymphocytes migrating out of the human gastric lamina propria following the loss of epithelial cells. *Gut* **1999,** 44, (3), 323-330.
2. McCaig, C.; Duval, C.; Hemers, E.; Steele, I.; Pritchard, D. M.; Przemeck, S.; Dimaline, R.; Ahmed, S.; Bodger, K.; Kerrigan, D. D.; Wang, T. C.; Dockray, G. J.; Varro, A., The role of matrix metalloproteinase-7 in redefining the gastric microenvironment in response to Helicobacter pylori. *Gastroenterology* **2006,** 130, (6), 1754-1763.
3. Holmberg, C.; Quante, M.; Steele, I.; Kumar, J.; Balabanova, S.; Duval, C.; Czepan, M.; Rakonczayjr, Z.; Tiszlavicz, L.; Nemeth, I.; Lazar, G.; Simonka, Z.; Jenkins, R.; Hegyi, P.; Wang, T.; Dockray, G.; Varro, A., Release of TGFbetaig-h3 by gastric myofibroblasts slows tumor growth and is decreased with cancer progression. *Carcinogenesis* **2012,** 33, 1553-1562.
4. Elias, J. E.; Gygi, S. P., Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nature methods* **2007,** 4, (3), 207-14.

Pooled sample	Primary interval (min)			Primary interval (min)			Primary interval (min)			Primary interval (min)		
1	20	-	21	35	-	36	50	-	51	65	-	66
2	21	-	22	36	-	37	51	-	52	66	-	67
3	22	-	23	37	-	38	52	-	53	67	-	68
4	23	-	24	38	-	39	53	-	54	68	-	69
5	24	-	25	39	-	40	54	-	55	69	-	70
6	25	-	26	40	-	41	55	-	56	70	-	71
7	26	-	27	41	-	42	56	-	57	71	-	72
8	27	-	28	42	-	43	57	-	58	72	-	73
9	28	-	29	43	-	44	58	-	59	73	-	74
10	29	-	30	44	-	45	59	-	60	74	-	75
11	30	-	31	45	-	46	60	-	61	75	-	76
12	31	-	32	46	-	47	61	-	62	76	-	77
13	32	-	33	47	-	48	62	-	63	77	-	78
14	33	-	34	48	-	49	63	-	64	78	-	79
15	34	-	35	49	-	50	64	-	65	79	-	80

Supporting Information Methods Table SM1. Fraction collection and pooling strategy for primary Methionyl-COFRADIC HPLC.

Fractions were collected at 1 min intervals between 20 and 80 min. These were pooled into 15 samples, such that the fractions shown in each of the 15 rows of the table were pooled together. In this way the number of samples is reduced whilst maintaining the peptide separation generated by reverse-phase HPLC, since the pooled fractions in the 15 samples each elute 15 min apart from each other.

Sample	Fraction interval (minutes)							
	Primary	**Secondary**	**Primary**	**Secondary**	**Primary**	**Secondary**	**Primary**	**Secondary**
1	20-21	8-18	35-36	23-33	50-51	38-48	65-66	53-63
2	21-22	9-19	36-37	24-34	51-52	39-49	66-67	54-64
3	22-23	10-20	37-38	25-35	52-53	40-50	67-68	55-65
4	23-24	11-21	38-39	26-36	53-54	41-51	68-69	56-66
5	24-25	12-22	39-40	27-37	54-55	42-52	69-70	57-67
6	25-26	13-23	40-41	28-38	55-56	43-53	70-71	58-68
7	26-27	14-24	41-42	29-39	56-57	44-54	71-72	59-69
8	27-28	15-25	42-43	30-40	57-58	45-55	72-73	60-70
9	28-29	16-26	43-44	31-41	58-59	46-56	73-74	61-71
10	29-30	17-27	44-45	32-42	59-60	47-57	74-75	62-72
11	30-31	18-28	45-46	33-43	60-61	48-58	75-76	63-73
12	31-32	19-29	46-47	34-44	61-62	49-59	76-77	64-74
13	32-33	20-30	47-48	35-45	62-63	50-60	77-78	65-75
14	33-34	21-31	48-49	36-46	63-64	51-61	78-79	66-76
15	34-35	22-32	49-50	37-47	64-65	52-62	79-80	67-77

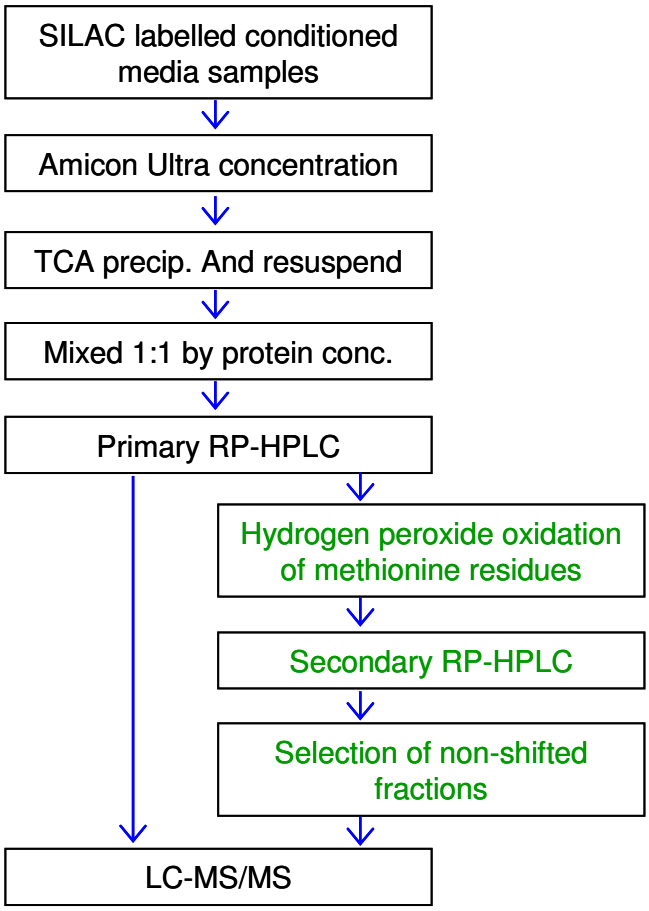
Supporting Information Methods Table SM2. Fraction collection strategy for secondary Methionyl-COFRADIC HPLC runs.

The 15 samples produced by pooling the primary fractions were each separated on secondary reverse-phase HPLC runs. The rows in the table show the primary fractions used to make up each sample. To the right of each of the primary fractions are the intervals at which corresponding secondary fractions were collected following methionine oxidation. Within the 10 min secondary intervals, 6 fractions were collected such that the first 2 fractions were each collected over a 3 min interval, and the remaining fractions were collected over a 1 min interval. Thus each of the 15 samples produced a total of 24 secondary fractions.

	TP	FP	FDR in %
Patient 1 (combined 'shotgun' and 'N-term' data)	6429	26	0.81
Patient 2 (combined 'shotgun' and 'N-term' data)	7559	27	0.71
Patient 1 (Met-COFRADIC)	18290	38	0.41
Patient 1 (N-term-COFRADIC)	5393	13	0.48

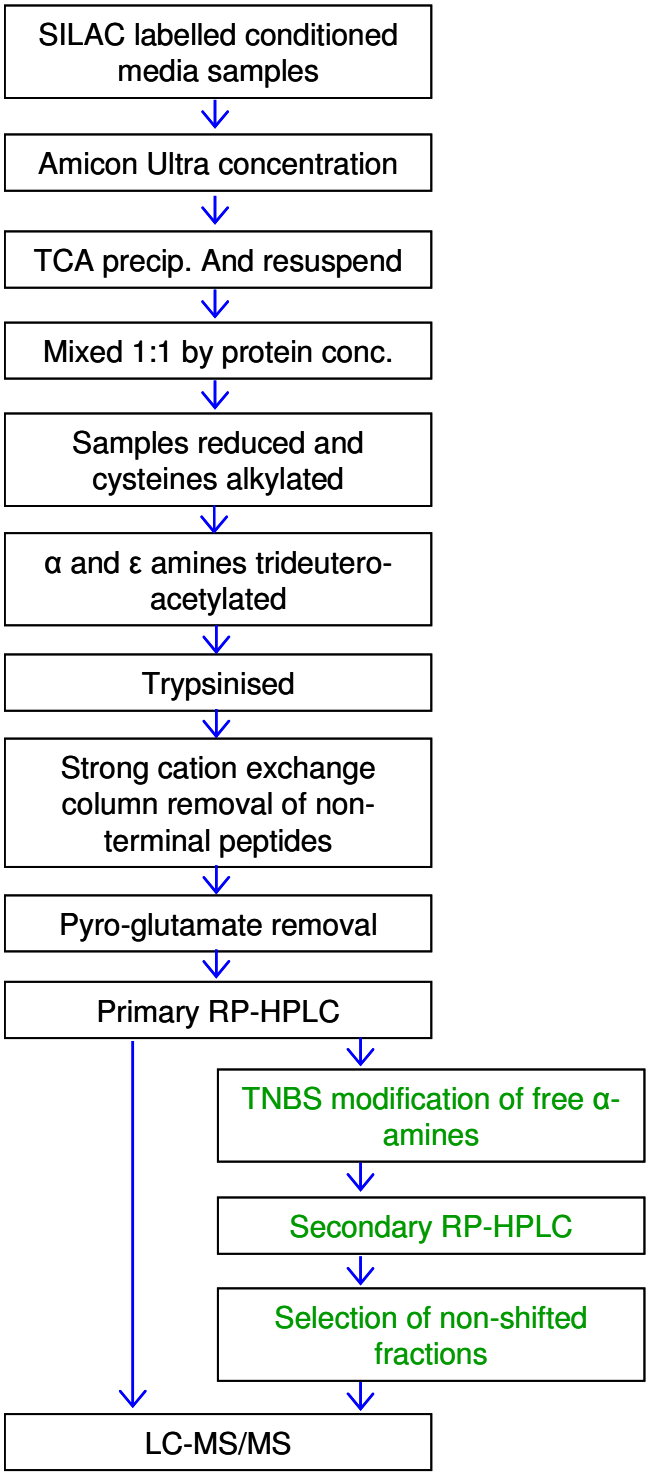
Supporting Information Methods Table SM3. The FDR was calculated as described by Elias et al.[4] Spectra were searched against a concatenated target-decoy database (the decoy database was a reversed form of the target database) to obtain the number of false positive (FP) and true positive (TP) identified spectra. The FDR was then calculated as (2*FP)/(TP+FP).

Methionyl-COFRADIC method



Supporting Information Methods Fig SM1. Experiment workflow for myofibroblast secretome mapping experiments. Green - Steps omitted in non-COFRADIC experiments.

N-terminal-COFRADIC method



Supporting Information Methods Fig. SM2. Experiment workflow for neo-N-terminal peptide indentification in myofibroblast secretomes. Green - Steps omitted in non-COFRADIC experiments.